

DETERMINATION OF GENETIC VARIATION BY USING ISSR MARKERS IN TOXIGENIC STRAINS OF *ASPERGILLUS* IN PADDY FROM TELANGANA STATE, INDIA

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ABSTRACT

Twenty four strains of *Aspergillus* species isolated from paddy (*Oryza sativa* L.) of four districts of Telangana State, India were tested for their genetic variation by using ISSR markers. Out of 50 ISSR primers, 16 primers produced scorable and reproducible banding patterns. Sixteen primers produced 388 band positions (loci) and out of these loci 370 loci amplified were polymorphic. Majority of the primers which produced polymorphic bands were GA or AG repeats followed by AC or CA repeats. Among the primers used, UBC-842 [(GA)₈ YG] produced maximum number of loci (and also polymorphic loci) while UBC-817 [(CA)₈A] produced least number of loci. The polymorphism (%) of the primers ranged between 88 (primer 809) and 100% (primers 808, 816, 834, 835 and 841). The PIC value ranged between 0.54 and 0.85. Most of the 3-prime single base anchored GA and AC repeats had higher PIC values (0.74-0.85) compared to 3-prime single base anchored AG and CA repeats (0.54-0.84). Using pooled ISSR data, a dendrogram was generated, (16 primers, 302 loci, 3042 bands) which divided 24 strains of *Aspergillus* into 7 major clusters based on their species and geographical origin.

KEYWORDS: Strains of *Aspergillus*, ISSR Markers, Genetic Variation

INTRODUCTION

Aspergillus, a ubiquitous fungus of diverse environments such soil, plant debris and indoor environment, plays an important role in the seed deterioration. Some species are capable of causing aspergillosis in animals including man (Barnes and Marr 2006; Deryck Damian Pattron 2006). The genus *Aspergillus* has well established identification parameters based on cultural and environmental characters and temperature relation (St-Germain and Summerbell 1996). *Aspergillus* is characterized by the production of aseptate conidiophore terminating in a vesicle, which bears conidiogenous cells (primary and secondary phialides and metulae) on which long chains of small, dry, single-celled conidia with varying pigmentation and ornamentation (Samson 1992). *A. fumigatus* is a thermotolerant fungus and grows well at temperatures over 40°C, while of *A. nidulans* and *A. glaucus* grow very slowly. These variations in growth pattern, help in species identification. *Aspergillus* section *Flavi* has attracted worldwide attention in view of toxigenic potential (Samson *et al.* 2000). This group includes *A. flavus*, *A. parasiticus* and *A. nidulans*. The methods for detecting these fungi and assessing their genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits. Such as restriction fragment length polymorphism (RFLP) (Soller and Beckman 1983; Rosewich *et al.* 1999) random

amplified polymorphic DNA (RAPD) (Williams *et al.* 1990; Neeraja *et al.* 2002), amplified fragment length polymorphism (AFLP) (Vos *et al.* 1995; Taheri *et al.* 2007), microsatellites or simple sequence repeats (SSRs) and inter simple sequence repeats (ISSRs) (Zietkiewicz *et al.* 1994; Hatti *et al.* 2010). Inter-simple sequence repeat-PCR (ISSR-PCR) is a simple, cost-effective, robust, multi-locus marker system which has been used in determining genetic variability among fungal pathogens (Menzies *et al.* 2003; Chadha and Gopalakrishna 2007). Primers based on a repeat sequence and the resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping. PCR products are separated on a agarose gel prior to autoradiographic visualization under ultraviolet light which concludes ISSR which is a better tool for genetic divergence and phylogenetic studies. Nagaoka and Ogihara (1997) also reported that the ISSR primers give more information than RAPD markers in wheat. We report here the possible use of ISSR-PCR in determining the genetic variability among *Aspergillus* strains of paddy.

MATERIALS AND METHODS

Isolation of the Fungal Strains Identification and Maintenance

Twenty four strains of *Aspergillus* were isolated from paddy (*Oryza sativa* L.) collected from rice mills of four districts of Telangana State, India and cultured on different media like, Czapek yeast agar (CYA), malt extract agar (MEA) and Czapek 20% sucrose. They were identified based on cultural and morphological characters as suggested by Raper and Fennell (1977); St-Germain and Summerbell (1996); Mathur and Kondgsdal (2003); Samson *et al.* (2007).

In all six species of *Aspergillus* were identified with following characters.

- ***Aspergillus Clavatus:*** It produces blue-grey colonies of 3-4 cm diameter in 10 days; conidial heads abundant, large, clavate (club shaped), blue-green when young, later slate-olive, 1-4 mm in length. Reverse of colony colorless at first, light-brown with age; conidia elliptical, smooth, somewhat heavy walled, light-green in mass.
- ***A. Flavus:*** Growing rapidly, conidial heads yellow when young, becoming dark-yellow green with age, in older cultures deep gray-green, reverse colourless to pale yellow-brown; conidial heads radiate, splitting into poorly defined columns, conidiophores arising separately from substratum. Conidia globose to sub-globose, conspicuously echinulate, yellowish-green.
- ***A. Parasiticus:*** Conidial heads first parrot-green to grass-green and finally dark dull yellow-green; reverse cream to light-drab; exudates limited; conidial heads loosely radiate; conidia globose to subglobose, coarsely echinulate, bright yellow-green in mass.
- ***A. Terreus:*** Colonies spreading on Czapek's medium, attaining 3.5-5.0 cm in diameter by 10 days. Plain or with radial furrows, velvety, floccose in some strains, cinnamon-buff to wood-brown, reverse dull-brown. Exudates amber coloured; conidial heads long columnar, compact with uniform diameter. Cinnamon-brown, conidiophores smooth, colourless vesicles hemispherical, dome like, globose to subglobose, phialides biserial, closely packed, metulae crowded, parallel, conidia, produced in chains, smooth, thick walled, hyaline, cells globose to ovate or even truncate.
- ***A. Niger:*** Conidial heads carbon-black to brownish-black. Reverse colourless to pale-yellow. Conidial heads large and black, conidiophores arising directly from the substratum, smooth, non-septate, thick-walled, vesicles globose, walls thick, commonly 45-75 µm in diam, conidia globose, spinulose with colouring substance, black,

some strains produced sclerotia.

- **A. *Fumigatus*:** Spreading rapidly, white at first becoming dull blue-green, velvety to floccose; reverse colorless to varying in shades, conidial heads columnar, compact, often densely crowded, conidiophores short, smooth, light-green, septate, gradually enlarging into a flask shaped vesicle; conidia globose to subglobose, green in mass, echinulate, sclerotia and cleistothecia absent.
- **A. *Nidulans*:** Dark cress green, abundant conidial heads, pinkish-cinnamon and form abundant cleistothecia, reverse purplish-red to very dark with age. Conidial heads slightly larger than typical representatives of species of *Aspergillus*. Conidial heads loosely radiate when young, later short columnar. Conidiophores light-brown, sinuous, smooth, occasionally septate, vesicles hemispherical, brown, conidia globose to sub-globose, rugulose, green in mass. The reverse is uncoloured to pale-yellow in most of the isolates.

DETECTION OF MYCOTOXINS

Aflatoxins produced by strains of *A. flavus* and *A. parasiticus* were extracted and detected by methods suggested by Stack and Pohland. (1975). Hundred ml of sucrose magnesium sulphate potassium nitrate yeast extract (SMKY, pH 6.0) broth was inoculated with a conidial suspension (1ml x 10⁶ spores) and incubated at 27±2°C for 10 days. At the end of incubation aflatoxins extracted with 300ml methanol and 200ml dichloromethane. The dichloromethane layer was collected and evaporated (in a water bath at approximately 60°C) using a rotary evaporator until approximately 10ml of the sample was left in the flask. Purification of the samples was carried out using the Vac-Elut system with Sep-Pak cartridges. The purified toxin was collected and dried under a gentle stream of nitrogen gas (Afrox, SA) using the Reacti-Vap Evaporating unit (Pierce, USA). The dried aflatoxin sample was analysed by TLC. Similarly other mycotoxins produced by other species of *Aspergillus* (*A. terreus*, *A. fumigatus*, *A. nidulans*, *A. niger* and *A. clavatus*) were also extracted and detected as précised in Table 1.

Table 1: Extractions and Detection of Different Mycotoxins by Different Species of *Aspergillus*

Species	Solvent System	Spray Reagent	Detection		Mycotoxin	Reference
			UV	Visible		
<i>A. flavus</i>	C:A (95: 5)	--	Blue and Green	--	Aflatoxin	Stack and Pohland (1975)
<i>A. parasiticus</i>	C:A (95: 5)	--	Blue and Green	--	Aflatoxin	Stack and Pohland (1975)
<i>A. terreus</i>	T: Ea: F (5: 4: 1)	Quantitative estimation	--	--	Terreic acid	Subramanian <i>et al.</i> (1978)
<i>A. fumigatus</i>	T:Ea:F (6:3:1)	H ₂ SO ₄	Brown	--	Gliotoxin	Adye and Mateles (1964)
<i>A. nidulans</i>	C:M:A (1:1:1)	AlCl ₃	Dull brick		Sterigmocysin	Ramakrishna <i>et al.</i> (1987)
<i>A. clavatus</i>	-	-	-	-	No toxin	-
<i>A. niger</i>	-	-	-	-	No toxin	-

C= Chloroform, A= Acetone, T=Toluene, Ea=Ethyl acetate, M=Methanol, F=Formic acid

DNA Isolation

The fungal DNA was isolated as suggested George *et al.* (1998). The isolates were grown in potato dextrose broth (PDB) under still culture at 27°C. At the end of 36 hours of growth, the mycelial mat was harvested, washed with sterile distilled water repeatedly and then squeezed in between layers of sterile blotting papers to remove excess water.

The mycelial mat was frozen in liquid nitrogen and ground into fine powder. About 30-40 mg of mycelial powder was suspended in 650 µl of extraction buffer (100 mM Tris, pH 8; 100 mM EDTA; 250 mM NaCl; and 1% sodium dodecyl sulfate, wt/vol), incubated at 65°C for 30-45 minutes. Cellular proteins were precipitated with 100 µl of potassium acetate (3 M potassium and 5 M acetate, pH 4.8), and then DNA was precipitated from filtrates using isopropanol. The precipitated DNA was dissolved in 100 µl of sterile distilled water. Two microliter of the DNA solution (50 ng) was used as template for PCR

PCR and Gel Electrophoresis

The sequence details of the ISSR primers were obtained from University of British Columbia website. Initially, 50 primers (UBC 801-UBC 850) were screened with a subset of samples. Sixteen primers which gave scorable banding pattern were used for analysis of all the samples. A single primer was used at a time for all the samples. Each reaction mix of 20 µl contained 2 µl of genomic DNA (50 ng), 1 µl of primer of 5 mM primer solution, 2 µl of 10x buffer (0.1 M Tris pH 8.3; 0.5 M KCl; 7.5 mM MgCl₂; 0.1% gelatin), 1 µl of 2.5 mM dNTPs and 1.0 unit of Taq polymerase. An additional of 1 µl of MgCl₂ (25 mM concentration) was added for better performance. PCR amplifications were performed in a thermal cycler (Applied Biosystems, USA) with the following conditions: initial denaturation at 94°C for 5 min, followed by 35/40 cycles of denaturing at 94°C for 1 min, annealing for at 50°C for 1 min extension at 72°C for 2 min and final extension at 72°C for 7 min. The amplification products were mixed with loading buffer (40% sucrose and 0.25% bromophenol blue) and resolved in 2% agarose gel in 1X TBE buffer under room temperature at a constant voltage of 90 V and detected by ethidium bromide staining. The molecular weight marker having 1Kbp ladder (Bangalore Genei Private Limited, India) was used for band sizing.

Preparation of Dendrogram and PCA Graph and Calculation of Primer Parameters

Each amplification product/band was considered as an ISSR marker allele. The reproducibility of the DNA profiles for all the isolates and for all the selected primers was tested by repeating the PCR and only reproducible amplicons were considered for analysis. Amplicons were recorded as present (1) or absent (0). The data were analyzed for different parameters like number of loci, number of polymorphic loci, polymorphism (%) and polymorphism information content (PIC) and primer resolving power (Rp). The PIC values were calculated using the formula

$$n$$

$$PIC = 1 - \sum_{j=1} P_{ij}^2$$

$$j=1$$

Where P_{ij} = frequency of the j^{th} pattern of the i^{th} band. Alternatively, PIC was calculated using online PIC calculator software (<http://www.genomics.liv.ac.uk/animal/Pic1.html>).

RESULTS

Twenty four fungal isolates (Table 2) were isolated from paddy seeds from four districts of North east of Telangana State were assigned to *Aspergillus flavus* (4), *A. parasiticus* (4), *A. niger* (4), *A. fumigatus* (3), *A. nidulans* (1) and *A. clavatus* (4) based on cultural and morphological characters. Sixteen ISSR primers out of 50 tested produced scorable and reproducible banding patterns. The details of primer wise parameters are presented (Figure 1) shows a representative banding pattern of 24 strains of *Aspergillus* using the primer UBC 807. Sixteen primers produced 388 band

positions (loci). Out of these, 370 loci amplified were polymorphic. Majority of the primers which produced polymorphic bands among the strains of *Aspergillus* were based on GA or AG repeats followed by AC or CA repeats (Table 3). Among the primers used, UBC-842 [(GA)₈YG] produced maximum number of loci (and also polymorphic loci) and UBC-817 [(CA)₈A] produced least number of loci. The polymorphism (%) of the primers ranged between 88 (primer 809) to 100% (primers 808, 816, 834, 835 and 841). The PIC value ranged between 0.54 and 0.85. Most of the 3-prime single base anchored GA and AC repeats had higher PIC values (0.74-0.85) compared to 3-prime single base anchored AG and CA repeats (0.54-0.84).

Table 2: Details of Twenty Four Isolates of Six Species of *Aspergillus*

Isolate Name	Location	Code	Seed Verity	DNA Order
<i>Aspergillus niger</i>	Adilabad	ADB	BPT-5204	1
<i>A. niger</i>	Karimnagar	KRN	MTU-1010	2
<i>A. niger</i>	Khammam	KMM	DRRH-3	3
<i>A. niger</i>	Warangal	WGL	BPT-5204	4
<i>A. flavus</i>	Adilabad	ADB	MTU-1010	5
<i>A. flavus</i>	Karimnagar	KRN	BPT-5204	6
<i>A. flavus</i>	Khammam	KMM	MTU-1001	7
<i>A. flavus</i>	Warangal	WGL	BPT-5204	8
<i>A. clavatus</i>	Adilabad	ADB	MTU-1010	9
<i>A. clavatus</i>	Karimnagar	KRN	BPT-5204	10
<i>A. clavatus</i>	Khammam	KMM	MTU-1010	11
<i>A. clavatus</i>	Warangal	WGL	BPT-5204	12
<i>A. terreus</i>	Adilabad	ADB	DRRH-3	13
<i>A. terreus</i>	Karimnagar	KRN	BPT-5204	14
<i>A. terreus</i>	Khammam	KMM	MTU-1001	15
<i>A. terreus</i>	Warangal	WGL	BPT-5204	16
<i>A. fumigatus</i>	Adilabad	ADB	MTU-1010	17
<i>A. fumigatus</i>	Karimnagar	KRN	BPT-5204	18
<i>A. fumigatus</i>	Khammam	KMM	MTU-1001	19
<i>A. nidulans</i>	Karimnagar	KRN	BPT-5204	20
<i>A. parasiticus</i>	Adilabad	ADB	MTU-1001	21
<i>A. parasiticus</i>	Karimnagar	KRN	BPT-5204	22
<i>A. parasiticus</i>	Khammam	KMM	DRRH-3	23
<i>A. parasiticus</i>	Warangal	WGL	MTU-1010	24

Table 3: Details of the Primers, Polymorphism and Banding Patterns of 24 Isolates of Six Species of *Aspergillus* by 18 ISSR Primers

ISSR Primers (UBC)	Primer Sequence	No. of loci	No. of Polymorphic loci	Polymorphism (%)	PIC
807	(AG) ₈ T	26	25	96.15	0.83
808	(AG) ₈ C	21	21	100.00	0.73
809	(AG) ₈ G	25	22	88.00	0.58
810	(GA) ₈ T	22	21	95.45	0.83
811	(GA) ₈ C	27	25	92.59	0.77
812	(GA) ₈ A	24	22	91.66	0.76
816	(CA) ₈ T	20	20	100	0.54
817	(CA) ₈ A	20	14	93	0.63
825	(AC) ₈ T	28	27	96	0.84
834	(AG) ₈ YG	23	22	95.65	0.73
835	(AG) ₈ YC	30	30	100.00	0.79
836	(AG) ₈ YA	22	22	100.00	0.84

Table 3: Contd.,					
840	(GA) ₈ YT	25	24	96.00	0.74
841	(GA) ₈ YC	25	25	100.00	0.85
842	(GA) ₈ YG	32	29	90.62	0.85
847	(CA) ₈ RC	23	21	91	0.56

Y = (C, T); R = (A,G)

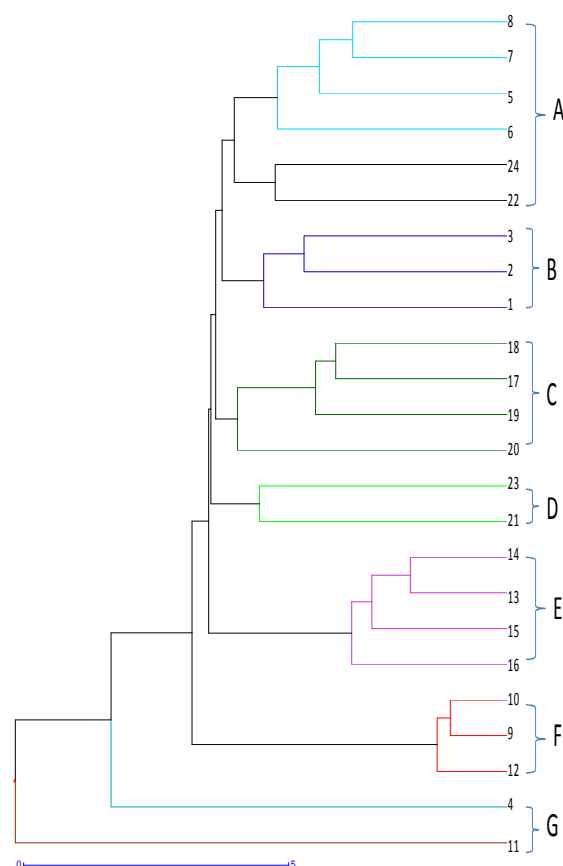


Figure 1: Cluster Analysis of ISSR Based PCR Patterns of *Aspergillus* Strains Collected from Telangana State

Using pooled ISSR data a dendrogram was generated, (16 primers, 302 loci, 3042 bands) which divided 24 *Aspergillus* strains into 7 major clusters (A-G) at 80% similarity (Table 4). The strain of *A. niger* [4] from Warangal district (mean genetic similarity 0.58) and the strain of *A. clavatus* [11] from the district of Khammam district (mean genetic similarity 0.74) were quite distinct from rest of the strains and also from each other. These species were also morphologically distinct from other strains. In general in the major cluster, the strains from same species or neighboring places were grouped together (Figure 1). Twenty four strains of *Aspergillus* were grouped into seven major clusters. In the main cluster A, the four [5,6,7,8] strains of *A. flavus* from four districts (ADB,KRN,KMM,WGL] and two strains of *A. parasiticus* [22,24] from two districts [KRN WGL] were grouped. The three strains of *A. niger* [1,2,3] were quite distinct and clustered separately. In the cluster C three strains of *A. fumigates* [17,18,19] from three districts [ADB,KRN,KMM] and one strain of *A. nidulans* from Karimnagar district [20] were grouped. Two strains of *A. parasiticus* [21, 23] from two districts [ADB, KMM] were clustered in the D. In the cluster of E four strains of *A. terreus* from four districts

[ADB,KRN,KMM,WGL] while three strains [9,10,12] of *A. clavatus* from three districts [ADB,KRN,WGL] clustered in the F. One strain [4] of *A. niger* [WGL] and one strain [11] of *A. clavatus* [KMM] were grouped in cluster G.

Table 4: Similarity Matrix Generated for Different Strains of Six Species of *Aspergillus* Using ISSR-PCR Based Primers

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1																							
2	0.4	1																						
3	0.5	0.5	1																					
4	0.5	0.4	0.7	1																				
5	0.4	0.3	0.4	0.4	1																			
6	0.4	0.3	0.4	0.4	0.6	1																		
7	0.4	0.3	0.4	0.4	0.7	0.6	1																	
8	0.3	0.3	0.3	0.3	0.6	0.5	0.6	1																
9	0.4	0.3	0.4	0.4	0.4	0.6	0.4	0.4	1															
10	0.4	0.3	0.4	0.4	0.4	0.6	0.4	0.3	1	1														
11	0.4	0.3	0.4	0.4	0.4	0.6	0.4	0.4	0.8	0.8	1													
12	0.4	0.3	0.4	0.4	0.4	0.6	0.4	0.3	0.9	0.9	0.8	1												
13	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	1											
14	0.4	0.3	0.4	0.4	0.3	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.9	1										
15	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.8	0.8	1									
16	0.4	0.3	0.4	0.4	0.3	0.4	0.3	0.3	0.3	0.3	0.4	0.3	0.7	0.7	0.8	1								
17	0.4	0.3	0.4	0.4	0.4	0.4	0.3	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.4	1							
18	0.4	0.2	0.3	0.3	0.3	0.4	0.3	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.8	1						
19	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.4	0.4	0.6	0.7	1					
20	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.5	1				
21	0.4	0.3	0.4	0.4	0.3	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.4	1			
22	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4	1		
23	0.4	0.3	0.4	0.4	0.3	0.4	0.3	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.3	0.4	0.5	0.3	1	
24	0.4	0.3	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.3	0.4	0.4	0.4	0.5	0.3	1

DISCUSSIONS

PCR is a simple, cost-efficient, robust, multi-locus marker method for determining genetic variability among the germplasm accessions of *Aspergillus*. Though, there are large number of reports of use of ISSRs for studying diversity of crop germplasm, its use in fungal diversity analysis is limited. In the present study, we have used ISSR-PCR to genetic variation of among different isolates of *Aspergillus*. Out of 50 ISSR primers screened 16 primers produced scorable and reproducible banding pattern. The number of loci varied from 15 (primer 816) to 32 (primer 842). Most of these primers produced polymorphic bands based on AG or GA repeats followed by AC or CA repeats. In an earlier study on analysis of genetic variation among the isolates of *Magnaporthe grisea* using ISSRs, Chadha and Gopalakrishna (2007) have also recorded that AG or GA repeats were more useful in differentiating the strains of *M. grisea*. The PIC value is important in determining the value of a primer to distinguish genotypes. The primers 807, 812, 825, 836, 841 and 842 exhibited higher PIC values. All these six primers individually could distinguish 22 out of 24 isolates of *Aspergillus* strains with distinct profile. Dendrogram generated from the pooled ISSR data (16 primers, 302 loci, 3042 bands) clearly grouped the isolates based on their species and geographical origin. The use of more number of loci from different ISSR primers increases the

accuracy of the grouping of the strains. The strain of *A. niger* (4) from Warangal district and the strain of *A. clavatus* (11) from Khammam district shared 41.22 percentage of similarity was quite distinct from other strain and also from each other. These species were also morphologically different from other strains. In general, in the major cluster, the strains from same species or neighboring places were grouped together (Figure 2). From the present studies it can be concluded that, ISSRs can satisfactorily cluster the *Aspergillus strains* on species and geographical basis.

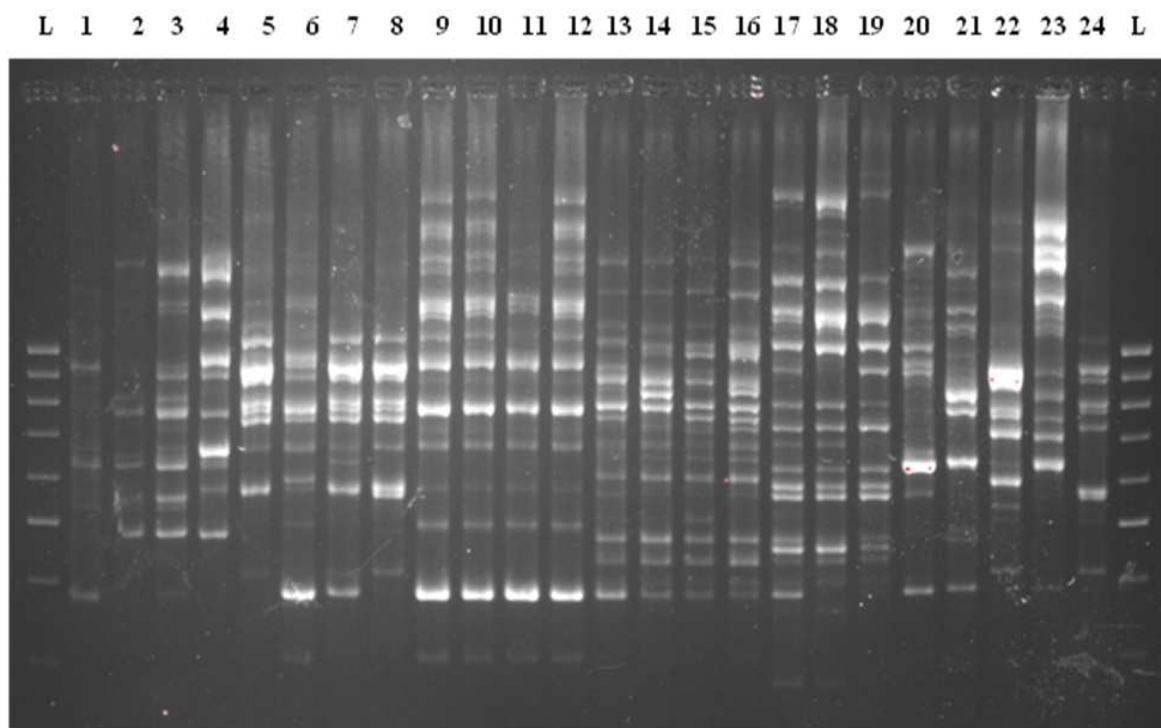


Figure 2: Amplification Pattern of Different Strains of Six Species of *Aspergillus* with ISSR Primer No 811

Amplification pattern of *Aspergillus* with ISSR primer No 811 on ethidium bromide stained 2% agarose gel (Marker: 100bp ladder, Genei, USA)

CONCLUSIONS

Among all the primers used Majority of the primers which produced polymorphic bands among the strains of *Aspergillus* were based on GA or AG repeats followed by AC or CA repeats; primers UBC-842 [(GA)₈YG] produced maximum number of loci (and also polymorphic loci) and UBC-817 [(CA)₈A] produced least number of loci. The primers 807, 812, 825, 836, 841 and 842 exhibited higher PIC values. All these six primers individually could distinguish 22 out of 24 isolates of *Aspergillus strains* with distinct profile. . The strain of *A. niger* (4) from Warangal district and the strain of *A. clavatus* (11) from Khammam district shared 41.22 percentage of similarity was quite distinct from other strain and also from each other. From the present studies it can be concluded that, ISSRs can satisfactorily cluster the *Aspergillus strains* on species and geographical basis.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to the Head, Department of Botany, Kakatiya University for encouragement and providing laboratory facilities and financially supported by UGC F.No: 36-131/2008 (SR), MRP New Delhi.

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